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TESTING OF RUGGEDIZED ANTIBODIES WITHIN A LATERAL FLOW IMMUNOASSAY

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14. ABSTRACT:
Antibody stability is a key quality parameter for a number of DoD customers who rely on effective and fielded hand-held immunoassays (HHAs). This project leverages antibody ruggedization efforts at U.S. Army Edgewood Chemical Biological Center (ECBC) to determine whether the ruggedization of detection antibodies translates into improved and more fieldable assays. Capitalizing on the work performed at ECBC in collaboration with the Defense Advanced Research Projects Agency, Antibody Technology Program and the Defense Threat Reduction Agency, Ruggedized Antibody Program, this effort was projected to develop a lateral flow immunoassay against the ricin toxin and increase thermostability that enlists the use of a smartCAR reader device, which is capable of communicating results with a smartphone. Incorporating previously ruggedized ricin antibodies into fieldable HHAs potentially makes these assays more amenable to forward-deployed hazard detection and eliminates (or greatly reduces) the current requirement for 4 ºC cold-chain logistics.

15. SUBJECT TERMS

Antibody	Ricin	Hand-held immunoassay (HHA)
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PREFACE

The work described in this report was supported by the U.S. Army Edgewood Chemical Biological Center 219 innovation project funds. The work was started in February 2015 and completed in September 2015.

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TESTING OF RUGGEDIZED ANTIBODIES WITHIN A LATERAL FLOW IMMUNOASSAY

1. INTRODUCTION

Immunoassays are the primary detection component of many of the Department of Defense (DoD)-fielded biological warfare agent (BWA) detection systems. Although antibody-based biosensors have been used by the DoD for decades, the lack of ruggedized reagents for use in these assays remains a significant issue. Lateral flow immunoassays (LFIs) are simple, hand-held devices designed to detect the presence (or absence) of a target antigen. They are compact, easily transported, and generally do not require additional reagents to achieve results. These biological detection assays are relatively inexpensive, easy to manufacture, and amenable to multiplexing; however, current LFI technology has certain limitations. These include a restricted shelf life, decreased sensitivity in austere environments, and a strong need for cold-chain logistics.[1] Warfighters, who depend the most upon these assays, are left at continued risk by these limitations. This project leverages previous antibody ruggedization efforts at U.S. Army Edgewood Chemical Biological Center (ECBC; Aberdeen Proving Ground, MD) that focused on antiricin antibodies. Ricin toxin is a glycoprotein produced by the castor bean plant, *Ricinus communis*, and is highly toxic. Ricin can cause death when given in sufficient quantities through systemic or inhalational routes of exposure.[2] Ricin was deployed as a weapon in the past and was famously used in the assassination of Georgi Markov during the Cold War.[3]

This project capitalizes on work performed by ECBC scientists in collaboration with the Defense Advanced Research Projects Agency (DARPA), Antibody Technology Program (ATP; Bethesda, MD) and the Defense Threat Reduction Agency (DTRA), Ruggedized Antibody Program (RAP). The DARPA ATP and DTRA RAP members established a test bed whereby "performers" could show off their capabilities to design enhanced antibodies. This project leveraged the products from the DTRA RAP, in which performers were given sequence data and raw material for one of two antiricin antibodies. The performers delivered a thermostable antibody derived from the parental antibody that they received.

In this project, we look to answer the question, "Does the ruggedization of detection antibodies translate into a more thermostable assay?" During the period of performance, Maxim Biomedical, Inc. (Rockville, MD) produced two lots of LFIs for the detection of the ricin toxin. The first LFI was prepared with parental antiricin antibodies, and the second was prepared with the thermostable version of those antibodies. All subsequent testing and evaluation of the LFIs were performed at ECBC.

2. MATERIALS AND METHODS

2.1 Antibodies

Several antibodies were evaluated to determine the ones that would work best as a pair in an LFI. Antiricin RIC-03-A-G1 (nonthermostable) was subcloned from an existing cell line in the Defense Biologics Products Assurance Office (DBPAO; BEI Resources; Manassas, VA**)** repository and grown in BD cell monoclonal antibody (Mab) media (BD Biosciences; San Jose, CA). Cell culture supernatant was collected, and the antibody was purified over protein A on the ÄKTAxpress system (GE Healthcare; Piscataway, NJ). Antiricin Mab2 (nonthermostable) was kindly provided by DBPAO members. The IGX2691 antibody was derived from RIC-03-A-G1 at AxioMx (Branford, CT). The AxioMx team also supplied IGX0207, which was derived from antiricin Mab2. AxioMx technicians rely on a proprietary graft and affinity maturation protocol to develop ruggedized immunoglobulin G (IgG) antibodies. APE 36633.03 (AnaptysBio; San Diego, CA) is also an IgG antibody that is derived from Mab2, as described in literature.[4,5] D12f101013 (Naval Research Laboratory; Bethesda, MD) was used to create a single-domain antibody (SdAb) independent of parental sequence data, as described in literature.[6]

2.2 NanoDrop Spectrophotometer

The NanoDrop ND-1000 (Thermo Fisher; Madison, WI) spectrophotometer was used to determine the concentration of the antibodies and measure the sample protein concentration using absorbance at 280 nm (A_{280}), which was influenced by the number of tryptophan and tyrosine residues in a given protein. For this reason, the extinction coefficient was used in conjunction with A_{280} to determine an accurate concentration. The concentration of each antibody was determined by taking the average A_{280} data divided by the extinction coefficient for each sample. Each reading required a 2 µL sample and was blanked using phosphate-buffered saline (PBS; Sigma-Aldrich; Saint Louis, MO). Readings were taken in triplicate. A positive control, bovine gamma globulin (BGG; Bio-Rad Laboratories, Inc.; Hercules, CA), was also tested to validate the instrument operation.

2.3 Experion Automated Electrophoresis System

The Experion (Bio-Rad) automated electrophoresis system was used to collect molecular weight and purity data. This system uses microfluidic technology to automate electrophoresis for protein analysis. The microfluidic chips, in conjunction with the Experion reagents, electrophoresis station, and software are designed to accomplish separation, staining, destaining, detection, and basic data analysis. The Experion Pro260 analysis kit uses engineered lower and upper internal alignment markers to provide accurate molecular weight sizing and quantitative protein analysis.[2] The Pro260 analytical software is also used to determine sample purity by calculating the percent mass of the separated proteins in a sample. For Experion analysis, each sample was standardized to a final concentration of 1 mg/mL by diluting in PBS. The control (BGG) and antibody samples were processed using a validated procedure included in the Experion Pro260 Analysis Kit, Rev. C. Briefly, a Pro260 microfluidic chip was prepared by adding 12 µL of Pro260 gel and gel stain to the designated wells. The chip was then placed on

the priming station and primed for 1 min at the medium (B) pressure setting. The sample was reduced with dithiothrietol (Sigma-Aldrich) and denatured in the kit-provided sample buffer at 95 °C before being applied to the primed chip. The instrument was operated using the Experion software; all samples were run in triplicate alongside one sample of the control (BGG) and Pro260 ladder (Bio-Rad). Analyses were performed using the Experion software.

2.4 Dynamic Light Scattering (DLS)

DLS was used to evaluate how the protein reacted in solution. DLS data indicate if a protein is aggregating in solution by measuring the polydispersity, hydrodynamic radius, and molecular weight of a sample. Prediction algorithms are employed by the software to produce a range of values for the protein under evaluation. For DLS analysis, five 20 µL aliquots of the antibodies and the control bovine serum albumin (Sigma-Aldrich) were placed into a quartz 384-well plate (Wyatt Technology Corporation; Santa Barbara, CA) and centrifuged for 2 min at (239 ×g) to remove trapped air bubbles from the samples. Mineral oil (Sigma-Aldrich) was applied to the top of each sample to prevent sample evaporation. The plate was placed into a DynaPro temperature-controlled plate reader (Wyatt Technology Corporation). Each well was scanned 10 times for 5 s each at 25 °C, and the average was calculated to provide measurements of polydispersity, hydrodynamic radius, percent mass, and molecular weight for each sample using the Wyatt Technology Dynamics software. The results of three wells were averaged together and reported.

2.5 LFI Pairs Testing

The DBPAO parental antibodies (nonthermostable) and the RAP produced (thermostable) antibodies were shipped to Maxim Biomedical. The Maxim Biomedical personnel tested all of the antibodies shown in Table 1 pair-wise to determine which antibody would be the best for capture and which would be the best for detection. Two nitrocellulose membranes were striped from the six ricin antibodies provided. Next, the antibodies were conjugated to colloidal gold. Each combination of striped membrane and conjugated gold were tested.

Table 1. Antibodies Used in This Study.

Striping	Gold Conjugation
A. Antiricin Mab2 (nonthermostable)	1. Antiricin Mab2
B. APE36633.03	2 APE36633.03
C. D12f101013	3. D12f102413
D. 1GX0207	4. 1GX0207
E. 1GX2691	5. 1GX2691
F. RIC-03-A-G1 (nonthermostable)	6. RIC-03-A-G1

Note: Each antibody was evaluated as a capture (striping) and detector (gold conjugation) antibody by Maxim Biomedical personnel.

After reviewing the initial results, the two best combinations were chosen: membrane A (antiricin Mab2) with gold 6 (RIC-03-A-G1) and membrane B (APE36633.03) with gold 5 (1GX2691). Further testing was conducted to optimize the lateral flow test with these two conditions. Once the lateral flow was optimized, bulk manufacturing of the striped membrane and gold conjugate proceeded.

2.6 LFI Testing

2.6.1 Limit of Detection (LOD) Estimation

The manufactured LFIs were shipped from Maxim Biomedical to ECBC and stored at 4 °C before testing was conducted. Initial testing to determine the LOD was performed using serial dilutions to find the optimum range for testing. All testing was done using the ricin A chain (Vector Labs; Burlingame, CA). Antigen concentrations ranged between 1000 and 0.1 ng/mL for the LOD estimation. LFI tickets were equilibrated to room temperature, and 100 uL of diluted antigen was applied dropwise to the test ticket window and left to incubate at room temperature for 15 min before reading was conducted on the CAMAG 4 scanner (Camag; Wilmington, NC). WINcats software (Camag) was used to perform quantitative evaluation of the generated densitometry data. Data was analyzed by plotting densitometry signal data against the concentration of antigen using GraphPad prism software (GraphPad Software, Inc.; La Jolla, CA). The LOD was estimated by analyzing signal versus concentration over a titration of ricin A chain. Data that fell within two standard deviations (2SD) of the cutoff value of 30 absorbance units was considered to be a positive signal.

2.6.2 Evaluation of Thermal Stability

A series of experiments was performed to determine how the two LFI lots would react when subjected to changes in temperature. The first experiment tested the thermostable and parental LFIs at 70 °C for 15, 30, 45, and 60 min before the antigen was applied at 25 ng/mL. The second set of experiments tested the thermostable and parental LFIs at 75 °C for 24 h before the antigen was applied at 10, 5, 2.5, and 1.25 ng/mL. Each trial was performed in triplicate. The results of all tickets were read after 15 min of incubation with antigen on the CAMAG 4 scanner, and data were analyzed with WINcats software and graphed in GraphPad.

3. RESULTS

3.1 Purification of RIC-03-AG1

The electronic gel image shown in Figure 1 was generated by the Bio-Rad Experion Pro260. This assay was used to determine the molecular weight and purity of all of the antibodies that were evaluated for this study.

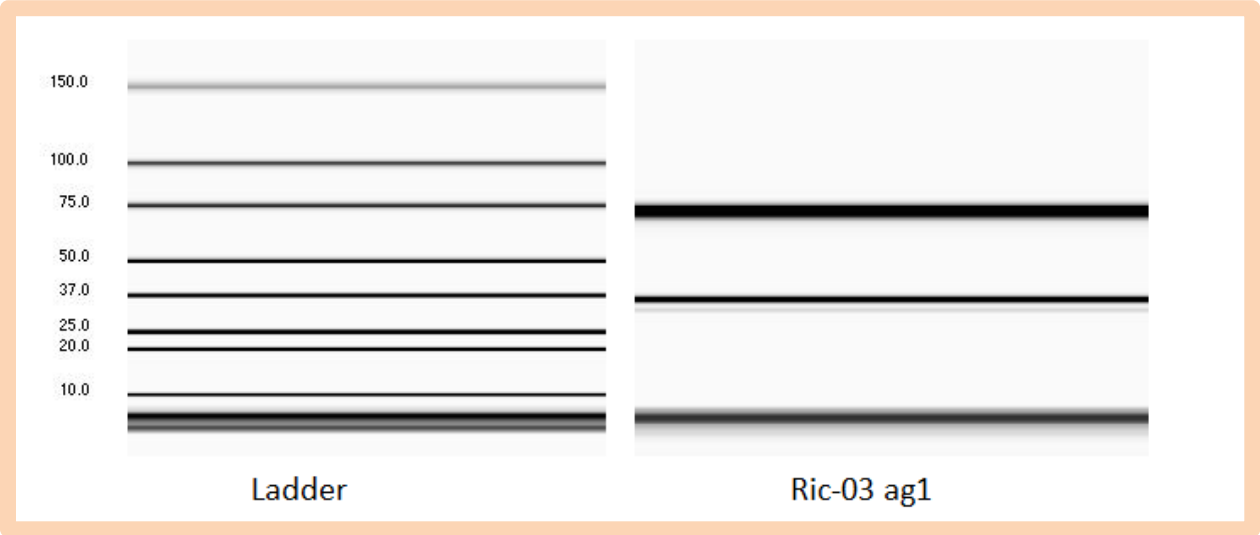

Figure 1. Molecular weight (MW) and purity of RIC-03-A-G1 produced and purified using ECBC biotechnology.

3.2 NanoDrop, Experion, and DLS

Table 2 shows that all antibody reagents used in this study were free from contaminants and were not aggregating at the time that the LFIs were produced. The specifications set for the DBPAO quality program are that an antibody should be >85% pure and >95% of the mass should have <37% polydispersity.

Table 2. Parametric Data from NanoDrop, Experion, and DLS
for All of the Candidate Antibodies Used in this Study

Sample Description ID	NanoDrop			Experion			DLS		
	A_{280} (OD)	ε280	Concentration (mg/mL)	MW (kDa) Heavy Chain	MW (kDa) Light Chain	Purity (%)	Radius (nm)	Mass %	Percent Polydispersity ≤36
Antiricin Mab2	0.56	1.4	0.41	63.1	26.7	93.9	5.6	99.8	
Δ03 APE 36633.03	0.51	1.4	0.37	65.3	27.3	99.8	5.2	99.9	
D12f-10-24-13	1.25	1.2	1.04	18.8	NA	94.4	1.9	100	Yes
IGX0207	0.43	1.4	0.31	60.3	28.9	95.3	5.8	99.9	
IGX2691	1.01	1.4	0.76	62.6	30.8	98.9	5.4	100	
Ric-03-AG1	0.75	1.4	0.55	72.2	35.6	97.2	5.6	100	

OD: optical density.
ε280: molar absorptivity at 280 nm.

3.3 Testing Completed at Maxim Biomedical

Pairwise testing performed at Maxim Biomedical indicated that the best combination for the nonthermostable LFI was antiricin Mab2 as the detector (gold) and RIC-03-A-G1 as the capture. For the thermostable LFI, the best combination was found to be APE36633.03 as the detector (gold) and 1GX2691 as the capture. Each combination of striped membrane and conjugated gold was tested, and its performance was evaluated by comparing intensity data. One lot of each thermostable and nonthermostable LFIs was manufactured and final bulk testing was performed.

Table 3 shows the data that were generated at Maxim Biomedical before the LFI lots were released to ECBC. The left-hand column shows the dilution factor of the antigen. The numbers listed under the two lots are the raw intensity data for the control stripe and the LFI test stripe. In the absence of antigen, the control stripe was positive and the test stripe was negative; this ruled out any false positives from buffer composition. The control stripe intensity data remained constant as the amount of antigen was diluted, whereas the test stripe decreased in intensity as the concentration of antigen was titrated down. This data indicated that both LFI lots were operating normally before they were shipped to ECBC.

Table 3. Final Bulk Testing: Lot No. R0001 (Nonthermostable) and R0002 (Thermostable)

Antigen Dilution	R0001		R0002	
	Control	Test	Control	Test
Negative	746	2	1025	1
1:100	857	1104	1089	1026
1:1000	761	783	1046	674
1:100K	808	391	1004	339

3.4 LFI Testing

The first experiment to be performed was a range-finding assay to determine the concentration range to target for future experiments. Initial experiments were performed starting at 1000 ng/mL, but the signal was maxed out until the concentration approached 100 ng/mL (data not shown). Figure 2 shows the results of subsequent testing that found the useful range was between 100 and 0.1 ng/mL. From these initial experiments, the LOD was estimated by choosing an antigen concentration that fell within 2SD of the cutoff value of 30 absorbance units. The cutoff value was chosen based on the conformance test plan that Camber Corporation (Oakridge, TN) follows for their LFI conformance testing. In this case, 0.75 ng/mL was considered to be a good estimate of LOD for test strips that were stored at 4 °C. For the purpose of this study, an estimate of LOD was sufficient because assay sensitivity was not the focus of this work.

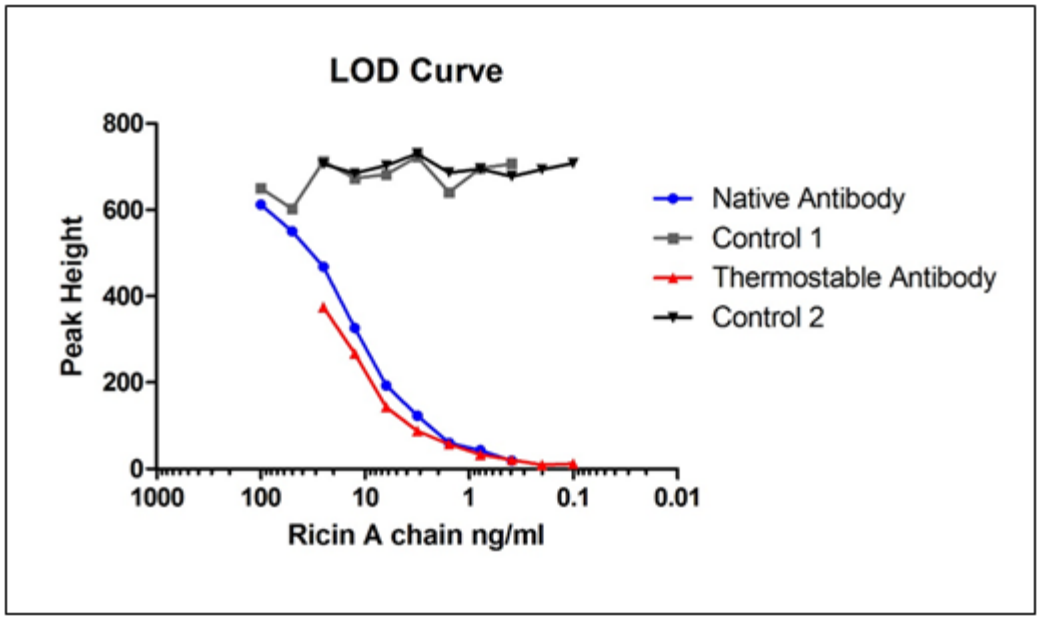

Figure 2. Graph of intensity data of LFIs exposed to a range of ricin A chain concentrations. (All of the LFIs were stored at 4 °C before use. Not much difference was seen between native and thermostable "ruggedized" LFIs when they were stored at a cold temperature. The black and grey lines represent LFI internal controls and are independent of antigen concentration.)

To determine if the ruggedization of detection antibodies translates into a more thermostable assay, the LFIs were exposed to high temperatures before being tested. In the first experiment, the LFIs were exposed to 70 °C for up to 1 h. The results indicated that there was not much difference between the native and thermostable LFIs (data not shown). Some research into the parental antibodies found that the melting temperature of one of the pair was 70 °C. Therefore, further experiments were performed at 75 °C. Initial experiments indicated that there was not much difference in the sensitivity until the concentration of antigen approached 10 ng/mL (data not shown). The remainder of the testing was performed between 10 and 0.1 ng/mL.

Figure 3 shows the difference in signal intensity between the native and ruggedized LFIs. The graph clearly shows that the thermostable antibodies in the ruggedized LFI retained their sensitivity between 10 and 0.1 ng/mL, whereas the native antibody LFI did not. When the raw intensity data was analyzed and the 4 and 75 °C data were compared to determine the percent difference in signal, the native LFI had an average of 71.4% decrease in signal intensity, whereas the ruggedized LFI had an average of 45.4% increase in signal intensity. The increase in signal over the 4 °C data could be because the thermostable antibodies remained intact when heated, instead of falling apart and losing activity. The thermostable antibodies acquired a certain "degree of freedom," which allowed them to become more flexible and better able to capture the antigen. More research is needed to understand the ways in which thermostable antibodies respond to heat stress.

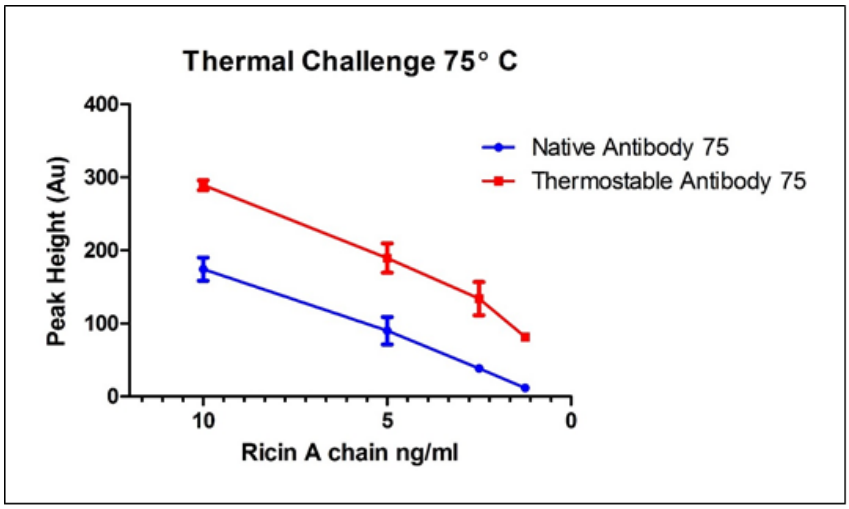

Figure 3. Difference in signal intensity between the native and ruggedized LFIs. (The blue line indicates that as the temperature was raised, the native antibody lost sensitivity, as compared with the thermostable antibody shown in red.)

4. DISCUSSION

Estimations by the Chemical Biological Medical Systems Joint Project Management Office at Joint Program Executive Office for Chemical and Biological Defense (Aberdeen Proving Ground, MD) showed that by eliminating the requirement for cold-chain storage, transport and deployment of antibody-based sensors would save the DoD approximately $10 million annually.[7] The data collected from this project could lead to the development of better antibody reagents for fielded assays. New and existing DBPAO antibody reagents presently in inventory could be ruggedized to improve affinity and stability for use with new materials that may be more practical, sustainable, and economical than the antibody strip and bead technology currently in use. This "ruggedization" effort eases cold-chain logistics making LFIs easier to store and transport in austere environments where traditional LFIs can be compromised by high temperatures. This effort was aimed at delivering a ruggedized portable LFI for the detection of ricin as a proof of principle. To further evaluate the efficacy of ruggedization, another pilot-scale project is recommended to determine the long-term stability of

these assays at different hold temperatures. Success of this effort will transition into the development of other antibody pairs that can be ruggedized to detect BWAs, thereby allowing for the development and fielding of better biological detectors.

5. CONCLUSIONS

We have demonstrated that engineered antibodies, which have been ruggedized to withstand higher temperatures, maintain the ruggedization when incorporated into hand-held assays. With the success of this effort, stakeholders such as DTRA and DBPAO should further support research and development of ruggedized reagents. This technology is specifically relevant on a case-by-case basis when the reagents that are used to field detection devices are known to be unstable. DBPAO (along with their DoD and non-DoD customers) will benefit from the potential cost savings projected by further research of the long-term stability and transition of these ruggedized assays. These cost savings will be realized through the reduction and removal of cold-chain logistics and are necessary for biosensor deployment.

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ACRONYMS AND ABBREVIATIONS

2SD	two standard deviations
A_{280}	protein concentration measured by using absorbance at 280 nm
ATP	Antibody Technology Program
BGG	bovine gamma globulin
BWA	biological warfare agent
DARPA	Defense Advanced Research Projects Agency
DBPAO	Defense Biologics Products Assurance Office
DLS	dynamic light scattering
DoD	Department of Defense
DTRA	Defense Threat Reduction Agency
ε280	molar absorptivity at 280 nm
ECBC	U.S. Army Edgewood Chemical Biological Center
IgG	immunoglobulin G
LFI	lateral flow immunoassay
LOD	limit of detection
Mab	monoclonal antibody
MW	molecular weight
OD	optical density
PBS	phosphate-buffered saline
RAP	Ruggedized Antibody Program
SdAb	single-domain antibody

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